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A STUDY OF THE GLOBULIN OF COTTONSEEDS.

XXIV. ISOLATION AND CHARACTERIZATION OF A GLYCOPEPTIDE OF THE 7S GLOBULIN

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UDC 547.962.5

From a pronase hydrolysate of the 7S globulin of cottonseeds, using a column containing Sephadex G-25 and paper chromatography, a glycopeptide has been isolated which contains in its molecule residues of aspartic acid, glucosamine, and mannose in a ratio of 1:2:5. It has been shown by an analysis of the products of the methylation of the glycopeptide that the carbohydrate chain has a branched structure.

In an investigation of the structure of the 7S globulin of cottonseeds we have shown that the Asn-129 residue of the polypeptide chain of one of the subunits of the protein is bound to an oligosaccharide chain consisting of glucosamine and mannose [1]. In order to study the structure of the carbohydrate moiety, the protein was hydrolyzed with pronase and the glycopeptide was isolated by ion-exchange chromatography on Dowex 50 × 2 cation-exchange resin, gel filtration on Sephadex G-25 (Fig. 1), and paper chromatography. An individual glycopeptide was obtained during a study of the amino acid and carbohydrate composition, which revealed the presence of residues of aspartic acid, glucosamine, and mannose in a ratio of 1:2:5. It is obvious that the 7S globulin is a glycoprotein with a N-acetylglucosaminylasparagine binding group [2-4]. The presence of acetyl groups in the glucosamine residue was shown by the appearance of the signals of protons at 1.88-1.96 ppm, while the PMR spectrum of the glycopeptide obtained was similar to that of soybean agglutinin [2] (Fig. 2).

To establish the structure of the oligosaccharide we used Hakomori methylation after N-acetylation, followed by separation of the permethylate by lipophilic gel filtration on Sephadex LH-20 [5-7]. In the investigation of the hydrolysate of the permethylate by TLC, three products were obtained, two of which (with R_f 0.41 and 0.66) were identified as 2,3,6-trimethyl- and 2,3,4,6-tetramethylmannoses in comparison with standard substances. The third product (R_f 0.24) was assigned to the dimethylmannoses. The results obtained, when account was taken of the intensities of coloration of all three derivatives, permitted the assumption that the carbohydrate chain has branching at a mannose residue.

Thus, the oligosaccharide of the 7S globulin of cottonseeds has a branched structure similar to other plant and animal glycoproteins with N-glycoside type of carbohydrate–protein bond.

EXPERIMENTAL

Isolation of the Glycopeptide. The protein (5 g) in 200 ml of 0.05 M phosphate buffer, pH 7.8, containing 0.3 M sodium chloride and $1.5 \cdot 10^{-3}$ M calcium chloride, was hydrolyzed with pronase (Merck). Incubation was carried out for 20 h at an enzyme:substrate ratio of 1:100, and then new portions of the enzyme (1:50) were added at incubation times of 45 and 24 h. The resulting mixture was centrifuged, and the supernatant was lyophilized. The product was dissolved in 50 ml of acetic acid solution with pH 3.25, and it was passed through

Institute of the Chemistry of Plant Substances, Academy of Sciences of the Uzbek SSR, Tashkent. Translated from Khimiya Prirodnykh Soedinenii, No. 2, pp. 215-217, March-April, 1981. Original article submitted October 9, 1980.

0009-3130/81/1702-0171$07.50

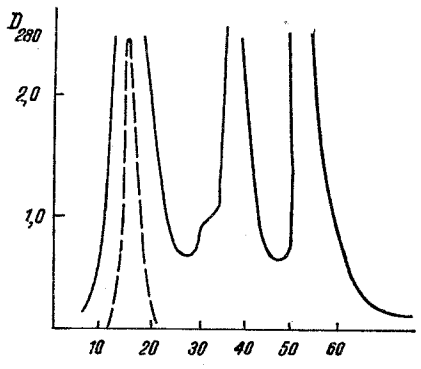

Fig. 1

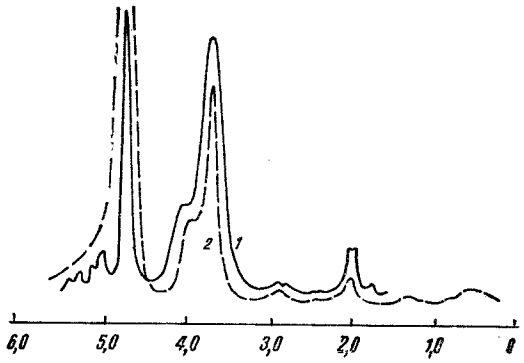

Fig. 2

Fig. 1. Separation of a pronase hydrolysate of the 7S globulin on a column of Sephadex G-25 (the dashed line shows the orcinol-positive fraction).

Fig. 2. PMR spectrum of the Asn-oligosaccharides of soybean agglutinin (1) and of the 7S globulin (2).

a column of Dowex 50 × 2, from which the orcinol-positive fraction was collected and lyophilized. This product was dissolved in 12 ml of 0.1 M ammonium bicarbonate solution and was deposited on a column of Sephadex G-25 (superfine, 2.5 × 55 cm, rate of elution 7 ml/h, 3.5 ml fractions).

The orcinol-positive fraction contained, according to paper chromatography, four ninhydrin-positive components, which were separated preparatively on FN-17 paper in the butan-1-ol—pyridine—acetic acid—water (15:10:3:12) system. Elution was performed with 0.1 M ammonium bicarbonate. Yield 40 mg (about 1%).

Quantitative Determination of Glucosamine. An aliquot (0.2 ml) of a solution of 0.8 mg of the glycopeptide in 0.8 ml of 6 N HCl was heated at 110°C for 24 h to hydrolyze it in order to determine its amino acid composition, and the remainder was diluted to 2 N HCl and hydrolysis was performed at 110°C for 16 h in order to determine glucosamine. The analysis was performed on an LKB 4101 analyzer.

Determination of Mannose. The glycopeptide (3 mg) was hydrolyzed in 5 ml of 3 N HCl at 100°C for 3 h, and the products were determined by GLC in the form of the TMS derivatives [8] on a Tsvet-4 instrument with a flame-ionization detector using a column (2 m × 4 mm) containing 5% of SE-30 on Chromaton N-AW (0.2-0.25 mm) at 170°C with a rate of flow of helium of 50 ml/min.

The PMR spectrum was recorded on a C-60HL, 60 MHz, instrument with HMDS as standard (internal), using 10 mg of the glycopeptide in 0.3 ml of D_2O.

N-Acetylation of the Glycopeptide [5]. At room temperature, 1.5 ml of acetic anhydride was added over an hour to a solution of 10 mg of the glycopeptide in 2 ml of 4.5 M sodium acetate. Then 40 ml of water was added and the mixture was heated at 100°C for 10 min, after which it was desalted on a column of Dowex 50 × 2 in the H^+ form (1.2 × 30 cm), eluted with water (150 ml), and lyophilized. The product was kept over P_2O_5 for 72 h.

Hakomori methylation [6] was carried out in a current of nitrogen. A solution of 10 mg of the substance in 10 ml of dimethyl sulfoxide was stirred for 1 h, and then 10 mg of sodium hydride was added in portions over 1 h followed similarly by 5 ml of methyl iodide. The reaction was performed for 5 h, and then the mixture was deposited on a column of Sephadex LH-20 (1.6 × 40 cm) in chloroform [7]. The rate of elution was 4.5 ml/h and the fraction volume was 2.2 ml, the glycopeptides being detected from the reaction with orcinol. The product was freeze-dried and was then dried further over P_2O_5. Completeness of methylation was confirmed by the insignificant absorption in the 3220-3700 cm^{-1} region (the IR spectrum of the syrupy product was recorded on a UR-20 spectrometer).

Hydrolysis of the Permethylate [7]. The permethylate was kept in a tube containing 3 ml of 90% formic acid at 100°C for 2 h and the mixture was then evaporated at 40°C and the residue was hydrolyzed in 5 ml of 0.3 N HCl at 100°C for 12 h. The mixture was evapo-

rated and chromatographed on Silufol plates (7 × 15 cm) in methyl ethyl ketone saturated with 1% aqueous ammonia, the spots being revealed with aniline phthalate. 2,3,6-Trimethylmannose and 2,3,4,6-tetramethylmannose were used as markers. The intensity of the spot of a dimethylmannose was approximately half that of the spots of the trimethyl- and tetramethylmannoses.

SUMMARY

A glycopeptide containing residues of aspartic acid, glucosamine, and mannose in a ratio of 1:2:5 has been isolated from a pronase hydrolysate of the 7S globulin of cottonseeds, and it has been shown that the mannose chain has a branched structure.

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SYNTHESIS OF 15-CARBOXY-(—)-KAURENES

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UDC 547.597:581.14

From the readily available (—)-kaurene and 6β-acetoxy-18-methoxycarbonyl-(—)-kaurene the corresponding 15-carboxy derivatives have been synthesized. In biotests on the elongation of wheat coleoptiles and the introduction of the synthesis of α-amylase in isolated wheat endosperms, these compounds do not exhibit gibberellin-like activity.

Among growth substance with gibberellin-like activity, considerable interest is presented by helminthosporins — substances with the carbon skeleton of the natural bicyclic sesquiterpenoid helminthosporol [1]. A characteristic feature of the structure of these compounds is the presence in their carbon skeleton of a bridge fragment of the bicyclo-[3,2,1]octane type that is characteristic of gibberellins and their biosynthetic precursors, (—)-kaurenes. In recent years, analogs of helminthosporol and helminthosporic acid have been synthesized many of which possess a considerable gibberellin-like activity [2]. The most active of them have proved to be compounds containing a carbonyl or a carboxyl group in the α position to a nodal carbon atom.

In view of this, and in connection with a further study of the dependence of the activity of this group of substances on their structure, we have synthesized two new 15-carboxy derivatives of (—)-kaurene (XIII and XIV). For this purpose we used (—)-kaurene (I) and 6β-acetoxy-18-methoxycarbonyl-(—)-kaurene (II), obtained previously by the methylation of stachysic acid [3].

The method of synthesizing compounds (XIII) and (XIV) was similar to that proposed previously for helminthosporol derivatives [4], with the difference that to obtain indi-

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Translated from Khimiya Prirodnykh Soedinenii, No. 2, pp. 217-220, March-April, 1981. Original article submitted July 8, 1980.

0009-3130/81/1702-0173$07.50